UCLA
UCLA Previously Published Works

Title

Exopolysaccharide production in Ensifer meliloti laboratory and native strains and their effects on alfalfa inoculation.

Permalink

https://escholarship.org/uc/item/1803018m

Journal

Archives of microbiology, 202(2)

ISSN

0302-8933

Authors

Primo, Emiliano D
Cossovich, Sacha
Nievas, Fiorela
et al.

Publication Date

2020-03-01

DOI

10.1007/s00203-019-01756-3

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Archives of Microbiology
https://doi.org/10.1007/s00203-019-01756-3

ORIGINAL PAPER

Exopolysaccharide production in *Ensifer meliloti* laboratory and native strains and their effects on alfalfa inoculation

Emiliano D. Primo[1] · Sacha Cossovich[1] · Fiorela Nievas[1] · Pablo Bogino[1] · Ethan A. Humm[2] · Ann M. Hirsch[2,3] · Walter Giordano[1]

Received: 20 August 2019 / Revised: 7 October 2019 / Accepted: 22 October 2019
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract
Bacterial surface molecules have an important role in the rhizobia-legume symbiosis. *Ensifer meliloti* (previously, *Sinorhizobium meliloti*), a symbiotic Gram-negative rhizobacterium, produces two different exopolysaccharides (EPSs), termed EPS I (succinoglycan) and EPS II (galactoglucan), with different functions in the symbiotic process. Accordingly, we undertook a study comparing the potential differences in alfalfa nodulation by *E. meliloti* strains with differences in their EPSs production. Strains recommended for inoculation as well as laboratory strains and native strains isolated from alfalfa fields were investigated. This study concentrated on EPS-II production, which results in mucoid colonies that are dependent on the presence of an intact *expR* gene. The results revealed that although the studied strains exhibited different phenotypes, the differences did not affect alfalfa nodulation itself. However, subtle changes in timing and efficacy to the effects of inoculation with the different strains may result because of other as-yet unknown factors. Thus, additional research is needed to determine the most effective inoculant strains and the best conditions for improving alfalfa production under agricultural conditions.

Keywords Alfalfa · *Sinorhizobium meliloti* · *Ensifer meliloti* · Exopolysaccharides · Symbiosis · Biofertilization

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage legumes for agriculture because it is well adapted to diverse environmental conditions (Avci et al. 2013). Consequently, it has become an important crop in many parts of the world including the Pampean region of central Argentina (Garcia et al. 2014) and also California (Geisseler and Horwath 2016). Although legume agriculture has expanded in Argentina, it has been recently influenced by a dramatic increase in soybean cropping (Caviglia and Andrade 2010), leading to a decrease in the land area allocated to forage crops such as alfalfa. As a result, forage crop systems based on the use of double crops where summer crops are used for silage and winter crops for pasture have become increasingly common (Abdelhadi et al. 2004; Arelovich et al. 2011). Nevertheless, the increasing demand for livestock feed in Argentina is negatively affected by suboptimal forage production levels, which is attributed to low nitrogen (N) fertilization rates (Agnusdei et al. 2010). Therefore, an increase in the efficiency of animal food production is needed to meet the growing demand within the constraints of the land area currently cultivated (Evans 1993).

Biofertilization of legume seeds with nitrogen-fixing bacteria collectively known as rhizobia started many years ago. Collectively, the legume-rhizobia symbiosis contributes over 40 million tons of N per year to agricultural systems (Herridge et al. 2008). Thus, the contribution of N by symbiotic fixation via the legume-rhizobia symbiosis is the most important source of N in agroecosystems (Ohyama 2017).

Communicated by Erko Stackebrandt.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00203-019-01756-3) contains supplementary material, which is available to authorized users.

✉ Walter Giordano
wgiordano@exa.unrc.edu.ar

[1] Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

[2] Department of Molecular, Cell and Developmental Biology, University of California-Los Angeles, Los Angeles, USA

[3] Molecular Biology Institute, University of California-Los Angeles, Los Angeles, USA

Published online: 03 November 2019

Enhancing the effectiveness of rhizobial inocula is desirable because bacteria can alleviate the requirement for synthetic N fertilizers that are economically and environmentally costly. In Argentina, a high percentage of legumes including soybeans, peanuts and alfalfa are inoculated (Vicario et al. 2015; López et al. 2018). However, very little information on the biological significance of biofertilization for *Medicago sativa* (alfalfa) is available.

E. meliloti is a soil-inhabiting N-fixing Alphaproteobacterium, which under conditions of N limitation, establishes a root nodule symbiosis with legume plants, especially alfalfa, but also with other *E. meliloti* host legumes such as *Melilotus alba* (Giordano and Hirsch 2004). As a result of this interaction, the bacteria provide ammonia to their hosts and receive nutrients, mainly carbohydrates, from them (Jones et al. 2007). The process of symbiotic N fixation is carried out in specialized root structures, i.e. nodules. The interaction of the rhizobia and legume plants is highly host-specific (Denarié et al. 1996), which is one of the many reasons agricultural legume seeds are coated with specific rhizobial inoculants. While rhizobia were once thought to be the only N-fixing inhabitants of legume nodules, other bacteria, which are distinct from rhizobia, are often isolated from surface-sterilized nodules obtained from soil, thus revealing the existence of a phytomicrobiome where the interaction among the individuals is not only complex but also likely to affect the behavior and fitness of the host plant (Martíınez-Hidalgo and Hirsch 2017). Previously, we reported on the complexity of the bacterial communities isolated from alfalfa rhizospheric soils in the agricultural region of the Argentinean Pampas (Bogino et al. 2013), but whether these or nodule microbiome bacteria influence the symbiosis is as yet unknown.

Rhizobial surface components, especially exopolysaccharides (EPSs), flagella, and lipopolysaccharides (LPSs), as well as bacterial and environmental signal molecules play important roles in symbiosis, particularly in the formation of metabolically active root nodules (Hirsch 1999). These molecules are also crucial for rhizobial biofilm formation (Fujishige et al. 2006; Rinaudi et al. 2006; Rinaudi and Giordano 2010) and for attachment to legume roots (Hirsch et al. 2009). Strains of *E. meliloti* produce two different EPS molecules: succinoglycan (EPS-I) and galactoglucan (EPS-II) (Fraysse et al. 2003). Each has a distinct role in the effectiveness of the symbiosis.

EPS-I, the better-understood and perhaps more symbiotically significant EPS, is required for invasion of alfalfa roots by *E. meliloti* strain Rm1021 (Reuber and Walker 1993). Mutations affecting EPS-I biosynthesis result in a variety of developmental abnormalities such as the formation of ineffective nodules (Fraysse et al. 2003). EPS-I also protects bacteria from various environmental stresses such as desiccation and may also have a signaling role in the establishment of the symbiosis (González et al. 1996), as shown for the *Mesorhizobium loti* and *Lotus japonicus* N-fixing associations (Kawaharada et al. 2015). Some conditions, for example, limitations of N and sulfur, high phosphate concentrations, and hyperosmotic stress stimulate EPS-I synthesis, whereas others such as phosphate starvation induce EPS-II production (Janczarek et al. 2014). EPSs are produced in two different forms, of high and low molecular weights, and are termed HMW and LMW, respectively. The LMW fraction is an active biological form of EPS that is essential for successful infection of leguminous plants that establish indeterminate-type nodules (Skorupska et al. 2006).

Under controlled laboratory conditions, Rm1021, a wild-type strain of *E. meliloti*, synthesizes detectable quantities of EPS-I but does not synthesize EPS-II. Synthesis of EPS-II in Rm1021 can be induced by environmental conditions and mutations, for example under low-phosphate conditions (Zhan et al. 1991) and in a *mucR* mutant (Keller et al. 1995). The presence of a functional *expR* gene is necessary to promote the synthesis of symbiotically active EPS-II in strain Rm8530, which has an intact *expR* and is termed *expR*$^+$ (Glazebrook and Walker 1989). However, strain Rm1021 carries an insertion sequence (IS) element within the *expR* gene, which prevents EPS-II synthesis (Pellock et al. 2002).

EPS-II production in *E. meliloti* facilitates autoaggregation, a mucoid phenotype, and biofilm formation (Rinaudi and González 2009; Sorroche et al. 2010). A positive correlation between biofilm formation and autoaggregation for *E. meliloti* strains isolated from alfalfa root nodules in the field led us to conclude that a functional EPS-II is needed for both bacterial-bacterial and bacterial-surface interactions (Sorroche et al. 2012). In addition, a mutation in LPS in the presence or absence of EPS-II leads to important changes in the adhesive properties of *E. meliloti* as well as in the symbiont's interaction with alfalfa (Sorroche et al. 2018).

We previously demonstrated by PCR analysis that the *expR* gene does not exhibit an obvious insertion in either indigenous *E. meliloti* strains isolated from Argentinean soils or the reference strain Rm8530 (Sorroche et al. 2012). However, relatively little is known about the efficacy of the inoculation response of alfalfa to many of the rhizobia used as inoculants. The objectives of this study were (1) to compare the genetic (*expR* gene structure) and biochemical (EPS production) features of two inoculant strains (*E. meliloti* B399 and B401) for alfalfa (Jozefkowicz et al. 2017a; b) with the laboratory reference strains (*E. meliloti* Rm1021 and Rm8530) as well as with indigenous strains (*E. meliloti* CU10 and SR9); and (2) to determine the physiological and symbiotic effects of inoculating alfalfa seedlings with these different *E. meliloti* strains. We also pursued an in silico analysis of several *E. meliloti* strains from various sources for the status of their *expR* genes to obtain more information about the importance of EPS-II for symbiotic nitrogen fixation.

Materials and methods

Bacterial strains and culture conditions

E. meliloti strains (Table 1) were grown under the conditions specified earlier (Sorroche et al. 2018). Antibiotics were used at the following final concentrations: streptomycin at 500 µg/ml, neomycin at 200 µg/ml, and gentamicin at 40 µg/ml. The strains were grown in minimal glutamate medium (MGM) for EPS determination (Marketon and González 2002).

Phenotypic assays

For visualization of mucoid or non-mucoid phenotypes, *E. meliloti* strains were streaked onto plates containing Luria Bertani (LB) medium. To observe the production of exopolysaccharides, the strains were grown on MGM agar with 125 µg/ ml Congo red (CR). A volume of 20 µl from a culture ($OD_{600} = 1$) was deposited on the agar medium containing CR. Plates were observed after 48 h of incubation at 30 °C.

DNA extraction

Colonies were suspended in 200 µl TE (Tris–EDTA) buffer, pH 8.0. DNA extraction was performed using Genomic DNA Purification Kit no. K0512 (Fermentas Life Sciences, Glen Burnie, MD, USA), following the manufacturer's instructions.

PCR amplification of *expR* gene

The *expR* gene was amplified from the genomes of the *E. meliloti* strains compiled in Table 1. Two specific primers were used for amplification of the *expR* region: Rm*ndv*A5´out (5´-CGAGGAGATCCTGCCCGAG-3´), and Rm*pyc*5´out (5´-AGAGTGGCGTGAACATTCGG-3´) (Pellock et al. 2002). The PCR amplification procedure used was described by Sorroche et al. (2012).

EPS quantification

Quantification of EPS production was performed by the anthrone-sulfuric acid and glucose standard method to determine the concentration in the supernatant of bacterial cultures grown in MGM medium (Doherty et al. 1988) with minor modifications (Wells et al. 2007) following the procedure described by Sorroche et al. (2018).

Plant nodulation tests

The nodulation assays were tested by inoculation with the different strains. Seeds of the alfalfa (*Medicago sativa*) "IMPERIAL Group 8" or "Pampeana" cultivar from INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina) were surface-sterilized and the alfalfa nodulation tests were performed as previously described (Sorroche et al. 2018).

Statistical analysis

A randomized design was used for all experiments. Values shown are average values ± SD from three independent pairs of duplicate experiments. Data were subjected to Analysis of Variance (ANOVA) with multiple comparison variables using Tukey´s test, and the Kruskal–Wallis test was used to analyze non-normally distributed data. Both tests were considered to be significant at $p \leq 0.05$. The software program used was Infostat 1.0 (Dept. of Statistics and Biometry, Faculty of Agricultural Sciences, National University of Córdoba, Argentina).

Table 1 Bacterial strains used in this study

Strain	Relevant properties	References
Reference *E. meliloti* strains		
Rm1021	SU47 *str21 expR102::*IS*Rm*2011-1*(expR⁻)*	Meade et al. (1982)
Rm8530	SU47 *str21 expR101 (expR⁺)*	Glazebrook and Walker (1989)
Commercial *E. meliloti* strains		
B399	Recommended as inoculant strain by INTA	Jozefkowicz et al. (2017b)
B401	Recommended as inoculant strain by INTA	Jozefkowicz et al. (2017a)
Native *E. meliloti* strains		
CU10	Isolated from alfalfa plants growing in soil from UNRC campus *(expR⁺)*	Sorroche et al. (2012)
SR9	Isolated from alfalfa plants growing in soil from San Rafael field *(expR⁺)*	Sorroche et al. (2012)

Genome analysis

We utilized the IMG/MER Integrated Microbial Genomics and Microbiomes website of the Joint Genome Institute (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) and queried a number of *Sinorhizobium* (*Ensifer*) *meliloti* genomes from the database using the gene sequence of accession number DQ366275.1, which is annotated by the website as a *S. meliloti* strain 8530 N-acyl homoserine lactone receptor gene, but is used to detect the presence of *expR* (Sorroche and Giordano, 2012). Information presented in Table S1 was derived from ten different *E. meliloti* strains at the IMG/MER website and from the literature cited.

Results and discussion

EPS production by *E. meliloti* is essential for root nodule formation on alfalfa and for the establishment of an N-fixing symbiosis between the two partners. Specifically, the production of EPS-II by *E. meliloti* strains results in a mucoid colony phenotype, but strains that are unable to produce EPS-II display a dry phenotype (Pellock et al. 2002; Sorroche and Giordano 2012). The different *E. meliloti* strains used in this study are described in Table 1 and Fig. 1 and the data from the anthrone analysis are shown in Fig. 2. Reference strain Rm1021 and its parent strain Rm2011 (Table S1) established dry colonies as did *E. meliloti* B399 and B401, two strains recommended as inoculants. In contrast, the indigenous strains SR9 and CU10 and reference strain Rm8530 exhibited a highly mucoid colony phenotype (Fig. 1a). Previous studies in our laboratory showed that native *E. meliloti* strains isolated from root nodules of alfalfa plants grown in four different regions of Argentina, among them strains SR9 and CU10, developed highly mucoid colonies (Sorroche et al. 2012). Similar results have been obtained for rhizobia isolated from nodules of *Medicago* spp. and *Melilotus alba* growing in California (Fujishige et al. 2008; E.A. Humm, unpubl.)

An evaluation of the production of EPSs was made using CR staining. CR binds to neutral or basic polysaccharides including beta-D-glucans and cellulose as well as amyloid proteins (Kneen and Larue 1983). On the plate supplemented with CR, the dry colonies were lightly colored in the center and surrounded by a red halo. In contrast, the colonies from the mucoid strains were redder in the center than the dry strains and convex in shape (Fig. 1b). These results show that the lighter-colored, dry colonies were devoid of a CR-positive material whereas the mucoid cells were red due to CR staining. In *Rhizobium leguminosarum*, Kneen and Larue (1983) postulated that CR might be binding to polysaccharides in the capsule, which might explain the differences in color observed in the two colony types.

To demonstrate that EPS production is responsible for the highly mucoid phenotype, the concentration of total soluble EPSs produced by each strain was analyzed by carrying out the anthrone method for determination of total carbohydrates. When the values were adjusted to bacterial dry weight, EPS production was higher in strain Rm8530 compared with the other strains under study (Fig. 2). In addition,

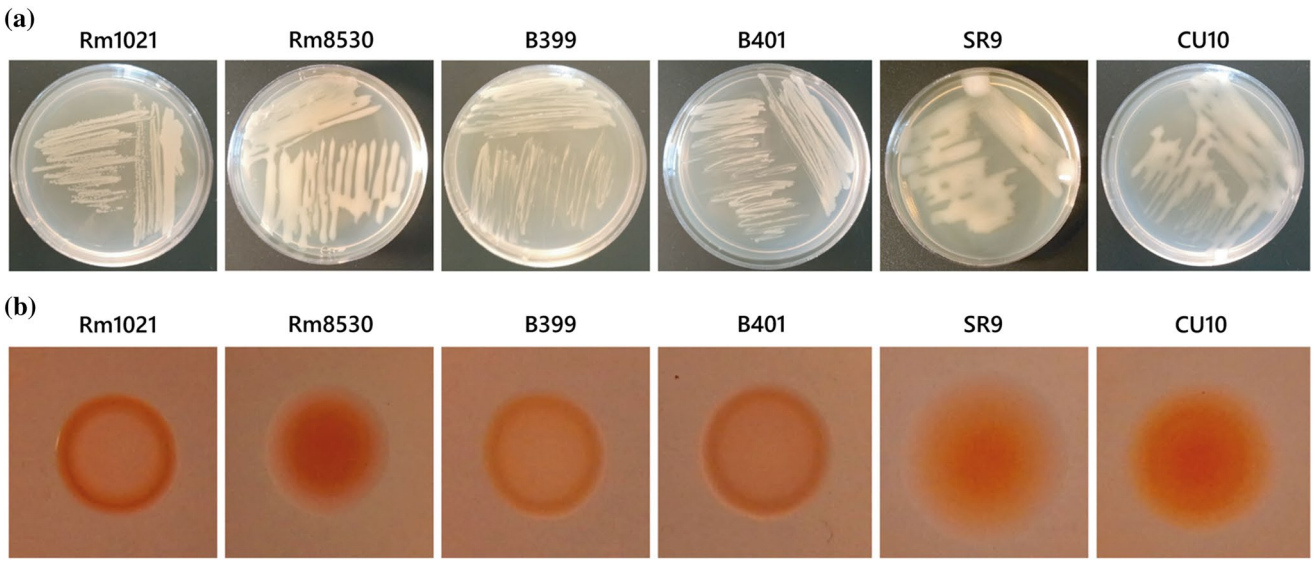

Fig. 1 Bacterial external features of *E. meliloti* strains. Overall colony appearance of Rm1021 (*expR*-); Rm8530 (*expR*+); B399 (*expR*-); B401 (*expR*-); SR9 (*expR*+); CU10 (*expR*+) on: **a** LB agar plates following 48 h of incubation; **b** MGM agar supplemented with 125 µg/ ml CR, where drops of inoculum were deposited. A single colony was photographed under white light after growth at 48 h at 30 °C

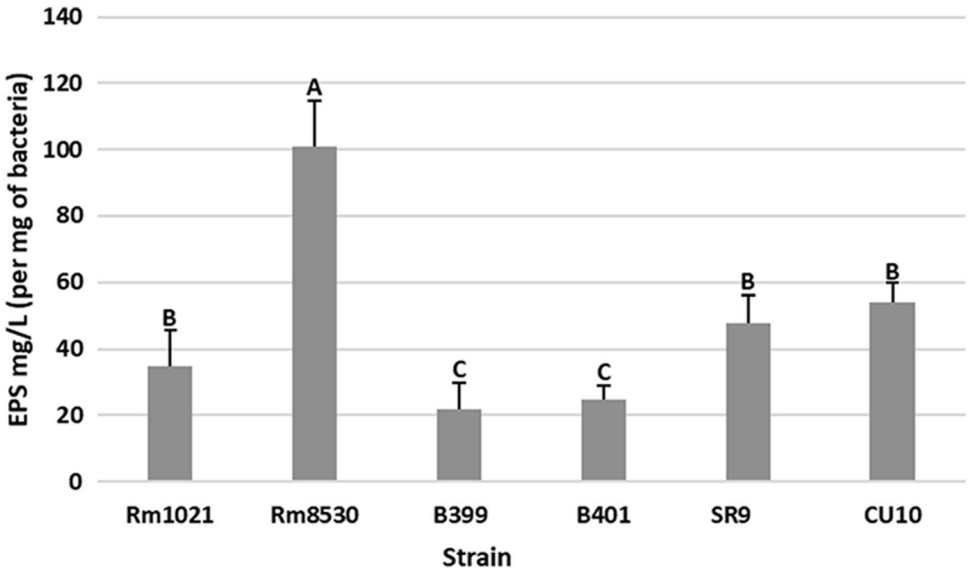

Fig. 2 EPSs production by *E. meliloti* strains. The average and standard deviation were calculated from three independent replicates. Different letters above bars indicate the EPSs values significantly differ among strains according to Tukey's test ($p \leq 0.05$)

the EPS levels in the indigenous strains SR9 and CU10 were higher than in the inoculant strains. However, for strain Rm1021, no statistically significant difference was observed from the indigenous strains, SR9 and CU10, although both exhibited more EPS production than the inoculant strains. For B399 and B401, the strains recommended for use as inoculants for alfalfa, EPS levels were similar to each other, but the values were significantly lower when compared to the other strains. These observations suggest a positive correlation between EPS production and colony morphology in these rhizobia strains.

The regulator ExpR controls the transcription of the *exp* genes involved in the production of symbiotically active EPS-II. To determine whether an IS in the *expR* locus was correlated with the differences in colony morphology and EPS production, we amplified the *expR* ORF with specific PCR primers designed to amplify the sequence of this gene. Figure 3 shows the PCR products obtained from native and reference strains. Based on size-fragment analysis, we found that *E. meliloti* Rm1021 had a nonfunctional gene as expected (Pellock et al. 2002; Simon et al. 1991). Its PCR product was 2.2 kb indicating that the *expR* ORF was disrupted by a copy of IS*Rm*2011-1, a 1319-bp IS element (Fig. 3, lane 2). *E. meliloti* Rm2011, the parent strain of Rm1021, also has an insertional mutation in the *expR* gene based on in silico analysis (Fig. 4). Furthermore, from a PCR of *E. meliloti* Rm8530, we obtained a 0.9 kb fragment, which corresponds to a functional *expR* gene (Fig. 3, lane 3) based on the agreement with the size predicted for strain Rm41. Because the genome of Rm8530 is not available, we used in silico analysis to determine that Rm41 has an intact *expR* ORF as shown previously by Pellock et al. (2002) and in Fig. 4. On the other hand, in commercial strains *E. meliloti* B399 and B401, the presence of a 2.2 kb PCR

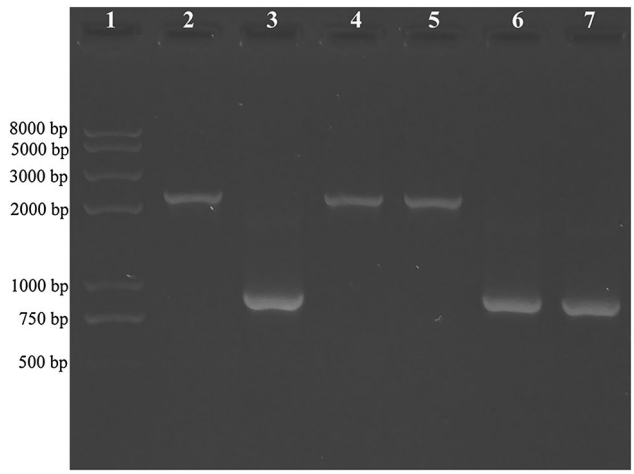

Fig. 3 Analysis of the *expR* locus. A 1% agarose gel showing the PCR products corresponding to the *expR* region from several *E. meliloti* strains. Lane (1) molecular marker (Plus II DNA Marker, TransGen Biotech); (2) Rm1021; (3) Rm8530; (4) B399; (5) B401; (6) SR9; (7) CU10

product indicated that the *expR* gene is interrupted (Fig. 3, lanes 4 and 5). These results are supported by in silico analysis of the genome of these strains (Jozefkowicz et al. 2017a; b) and other strains (Table S1).

A 0.9 kb fragment was detected in the native strains *E. meliloti* SR9 and CU10 (Fig. 3, lanes 6, 7), showing that there is no IS element similar to IS*Rm*2011-1, which interrupts the *expR* gene. However, finding a fragment of 0.9 kb does not necessarily confirm the presence of a functional *expR* gene for these strains. For example, Charoenpanich et al. (2015) showed that a single nucleotide polymorphism (SNP) or an insertion/deletion in the *expR* gene disrupts ExpR function, producing a dry colony phenotype.

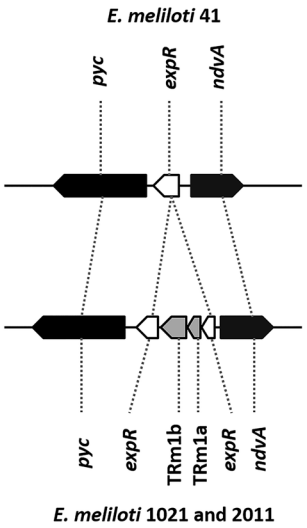

Fig. 4 Map of genes flanking *expR* in *E. meliloti* strains Rm1021/2011 and Rm41. In both Rm1021 and Rm2011, *expR* is interrupted by TRm1a and TRm1b, transposons in ISRm1, an insertion element. Rm2011 has two genes annotated as non-coding RNA sequences to the right of *expR*

Nonetheless, the strains SR9 and CU10 evaluated in this work are highly mucoid (Fig. 1a and b). This correlation of genotypic and phenotypic results suggests the presence of a functional *expR* gene in the native strains studied here (Charoenpanich et al. 2015). In addition, these phenotypes are consistent with the mucoid colony phenotype described for strains Rm8530 and Rm41, both of which are more mucoid than Rm1021 (Pellock et al. 2002). An analysis of the genomes of several *E. meliloti* strains in the IMG database indicated that an IS element is the exception rather the rule (Table S1). Only Rm1021 and its parent strain Rm2011 were observed to have the *expR* gene interrupted based on in silico evidence. Taken together, our results indicate that the IS*Rm*2011-1 insertion in the *expR* gene of strains Rm1021, B399, and B401 (Fig. 3) affected EPSs production and induced a non-mucoid phenotype, which most likely accounts for their dry appearances (Fig. 1a and b).

Because no significant progress in the production of improved N-fixing inoculants for alfalfa production has been made for some time (Jozefkowicz et al. 2017b), the final objective of this work was to determine whether *E. meliloti* could become more effective for inoculation. Therefore, a comparative analysis of inoculation with the different *E. meliloti* strains (indigenous, commercial, and reference strains described in Table 1) was performed. Each strain was inoculated on the roots of alfalfa seedlings. Although the total nodule number was slightly higher for the indigenous strain CU10 inoculum compared with the other strains (Table 2), none of the increases in nodule number were statistically different from one another at the end of the experiment. The plants inoculated with the reference Rm8530 and the indigenous B401 strains developed the fewest number of nodules, approximately 50% fewer than CU10. As expected, no nodules developed on the roots of any of the uninoculated (control) plants. Overall, all the nodules that were observed were effective. Nevertheless, these results indicate that rhizobial EPSs production of a particular inoculated strain does not correlate with total nodule number. Moreover, no statistically significant differences with regard to shoot and root fresh weight of alfalfa plants inoculated with different strains and among inoculated and non-inoculated plants were observed (Table 2). This might be attributed to the fact that these are relatively short-term experiments and were not of sufficient duration to show a significant effect on biomass production.

We previously reported that *E. meliloti* SR9 displayed high autoaggregation and biofilm formation phenotypes whereas CU10 had low autoaggregation and biofilm abilities (Sorroche et al. 2012). The present study's findings illustrate the variability in the nodulation abilities between these two strains, but overall, the total nodule number was lower for strain SR9 and higher for CU10 suggesting there may be a correlation between autoaggregation and biofilm capabilities. The above observations also suggest that rhizobial cell surface components such as EPS, in combination with bacterial signal molecules, are necessary for the adhesive interaction, but may not necessarily improve the symbiosis, at least based on these conditions and short-term experiments. More studies are needed. On the other hand, EPSs may help rhizobia adapt to stressful environmental conditions such as desiccation or may help to promote mixed biofilms that serve

Table 2 Nodulation assay[a]

Plant responses	Rm1021	Rm8530	B399	B401	SR9	CU10	Non-inoculated
Nodule number	3 ± 1.29^{ab}	2.5 ± 1.05 a	4.39 ± 1.6^{cd}	2.3 ± 1.36^{a}	3.88 ± 1.26^{bc}	5.53 ± 1.55^{d}	0
Nodulated plants (%)	84	87.5	88.44	95	95.45	90.9	0
Shoot fresh weight (mg)	25.5 ± 6.7	23.51 ± 6.56	29.88 ± 7.67	27.13 ± 7.30	28.14 ± 5.8	28.28 ± 5.5	25.86 ± 6.14
Root fresh weight (mg)	34.45 ± 9.34	32.14 ± 12.61	26.44 ± 7.77	27.36 ± 8.93	28.46 ± 8.66	35.40 ± 10.96	32.49 ± 10.32

[a]Average and standard deviation of at least three independent experiments with five replicates each are shown. Different letters above indicate statistically significant differences among average values ($p > 0.05$, Kruskal–Wallis test)

as consortia to improve nodule development and function. These consortia may become part of the complex microbiomes observed in nodules (Martinez-Hidalgo and Hirsch 2017). As mentioned earlier, many of the bacteria isolated from field-grown nodules are highly mucoid (unpubl. data). Experiments to discern the differences between rhizosphere and nodule microbiomes may answer this question.

Conclusions

Genotypic analysis as described here demonstrates that an insertion into *expR* in strains Rm1021, B399, and B401 (Fig. 3) is positively correlated with an effect on EPS production that results in a non-mucoid phenotype. However, the role of *E. meliloti* EPSs in early interactions with alfalfa roots has been extensively evaluated mostly from utilizing rhizobial model systems such as Rm1021. The role of EPSs in native *E. meliloti* strains is less studied compared to investigations using Rm1021 and its mutants. When we had previously investigated *E. meliloti* adsorption to alfalfa roots with indigenous strains mutated in EPS synthesis (Sorroche et al. 2012), we found that EPS-II partially inhibited rhizobial adhesion to plant roots and suggested that it occurred through a shielding effect. In this context, it is noteworthy that we have recently conducted nodulation assays on alfalfa using a lipopolysaccharide mutant of the model strain Rm1021. These data showed that a higher degree of attachment to roots by the LPS mutant did not result in either an increase of nodule number or of nodulated plants (Sorroche et al. 2018). Taken together, these observations lead us to conclude that although there might be a direct relationship between the production of rhizobial surface molecules, mucoid colony morphology, and rhizobial adhesion to plant roots, these phenotypes are not necessarily connected to the symbiotic response, which is primarily mediated by genes directly or indirectly involved in nodulation. We are continuing to investigate these phenomena.

Our results further indicate that native *E. meliloti* strains isolated from root nodules of alfalfa plants grown in Argentinean soils are potential alternatives to the currently utilized biofertilizers, most of which are produced from rhizobia that form dry colonies. We do not know at this time the exact mechanisms whereby nodulation is enhanced, but increased tolerance to environmental stress due to greater EPS-II production may be one possibility as is the ability to group with other soil microbes that promote symbiosis. Our results demonstrate that the indigenous strain CU10 is highly efficient in terms of nodulation despite having been previously described as very ineffective in adhesion capacity. The present data provide an important background for future investigations of alternative inoculant strains and conditions for improving alfalfa production.

Acknowledgements Grants from the Secretaría de Ciencia y Técnica de la UNRC, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Consejo Nacional de Investigaciones Científicas y Técnicas of the República Argentina (CONICET) supported this work. EP and SC have a fellowship from the CONICET. FN, PB, and WG are Career Members of CONICET. Our thanks are due to Dr. Nicolás Ayub for helpful suggestions and useful discussions. Support from a Shanbrom Family Fund grant supported research on symbioses in arid environments in the Hirsch laboratory. This research was funded by FONCyT Grant number [PICT 1528/15].

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

Abdelhadi LO, Lyons TP, Jacques KA (2004) Evaluating inoculants for forage crops in Argentine beef and milk grazing systems: effects on silage quality and system profitability. In: Lyons TP, Jacques KA (Eds.) Nutritional biotechnology in the feed and food industries. Alltech 20th Annual Symposium. Nicholasville, KY, USA pp. 171–177.

Agnusdei MG, Assuero SG, Lattanzi FA, Marino MA (2010) Critical N concentration can vary with growth conditions in forage grasses: implications for plant N status assessment and N deficiency diagnosis. Nutr Cycl Agroecosys 88:215–230

Arelovich HM, Bravo RD, Martínez MF (2011) Development, characteristics, and trends for beef cattle production in Argentina. Anim Front 1:37–45

Avci MA, Ozkose A, Tamkoc A (2013) Determination of yield and quality characteristics of alfalfa (*Medicago sativa* l.) varieties grown in different locations. J Amin Vet Adv 12:487–490

Bogino P, Abod A, Nievas F, Giordano W (2013) Water-limiting conditions alter the structure and biofilm-forming ability of bacterial multispecies communities in the alfalfa rhizosphere. PLoS ONE 8(11):e79614

Caviglia OP, Andrade FH (2010) Sustainable intensification of agriculture in the Argentinean Pampas: capture and use efficiency of environmental resources. Am J Plant Sci Biotechnol 3:1–8

Charoenpanich P, Becker A, Soto MJ, McIntosh M (2015) Quorum sensing restrains growth and is rapidly inactivated during domestication of *Sinorhizobium meliloti*. Environ Microbiol Rep 7:373–382

Denarié J, Debellé F, Promé JC (1996) *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65:503–535

Doherty D, Leigh JA, Glazebrook J, Walker GC (1988) *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. J Bacteriol 170:4249–4256

Evans LT (1993) Crop evolution. Adaptations and yield. Cambridge University Press, Cambridge

Fraysse NF, Couderc F, Poinsot V (2003) Surface polysaccharide involvement in establishing the *Rhizobium*-legume symbiosis. Eur J Biochem 270:1365–1380

Fujishige NA, Kapadia NN, De Hoff PL, Hirsch AM (2006) Investigations of *Rhizobium* biofilm formation. FEMS Microbiol Ecol 56:195–205

Fujishige NA, Jankaew K, Hirsch AM (2008) Biofilm formation in *Sinorhizobium meliloti*. Biology of plant-microbe interactions, Volume 6. In: Proceedings of the 13th International Congress on Molecular Plant-Microbe Interactions. Eds. Lorito M, Woo SL, and Scala F. ISBN 0965462552

Garcia AN, Ayub ND, Fox AR, Gomez MC, Dieguez MJ, Pagano EM, Berini CA, Muschietti JP, Soto G (2014) Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. BMC Plant Biol 14:248

Geisseler D, Horwath WR (2016) Alfalfa production in California. https://apps1.cdfa.ca.gov/FertilizerResearch/docs/Alfalfa_Production_CA.pdf. Accessed 16 Apr 2019

Giordano W, Hirsch A (2004) The expression of MaEXP1, a Melilotus alba expansin gene is up-regulated during the sweetclover-*Sinorhizobium meliloti* interaction. Mol Plant Microbe Interact 17:613–622

Glazebrook J, Walker GC (1989) A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell 56:661–672

González JE, York GM, Walker GC (1996) *Rhizobium meliloti* exopolysaccharides: synthesis and symbiotic function. Gene 179:141–146

Herridge D, Peoples M, Boddey R (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Hirsch AM (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. Curr Opin Plant Biol 2:320–326

Hirsch AM, Lum MR, Fujishige NA (2009) Microbial encounters of a symbiotic kind- attaching to roots and other surfaces. In: Emons AMC, Ketelaar T (eds) Root hairs. Plant cell monographs, 12th edn. Springer, Berlin, pp 295–314

Janczarek M, Rachwal K, Marzec A, Grzadziel J, Palusinska-Szysz M (2014) Signal molecules and cell-surface components involved in early stages of the legume–*Rhizobium* interactions. Appl Soil Ecol 85:94–113

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat Rev Microbiol 5:619–633

Jozefkowicz C, Brambilla S, Frare R, Stritzler M, Piccinetti C, Puente M, Berini C, Reyes Pérez P, Soto G, Ayub N (2017a) Stable symbiotic nitrogen fixation under water-deficit field conditions by a stress-tolerant alfalfa microsymbiont and its complete genome sequence. J Biotechnol 263:52–54

Jozefkowicz C, Brambilla S, Frare R, Stritzler M, Puente M, Piccinetti C, Soto G, Ayub N (2017b) Microevolution rather than large genome divergence determines the effectiveness of legume-rhizobia symbiotic interaction under field conditions. J Mol Evol 85:79–83

Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszyński A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, Vinther M, Andersen SU, Krusell L, Thirup S, Jensen KJ, Ronson CW, Blaise M, Radutoiu S, Stougaard J (2015) Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature 523:308–312

Keller M, Roxlau A, Weng WM, Schmidt M, Quandt J, Niehaus K, Jording D, Arnold W, Pühler A (1995) Molecular analysis of the *Rhizobium meliloti mucR* gene regulating the biosynthesis of the exopolysaccharides succinoglycan and galactoglucan. Mol Plant Microbe Interact 8:267–277

Kneen BE, LaRue TA (1983) Congo red absorption by *Rhizobium leguminosarum*. Appl Environ Microbiol 45:340–342

López SMY, Molina Sánchez MD, Pastorino GN, Franco MEE, García NT, Balatti PA (2018) Nodulation and delayed nodule senescence: strategies of two *Bradyrhizobium japonicum* isolates with high capacity to fix nitrogen. Curr Microbiol 75:997–1005

Marketon MM, González JE (2002) Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J Bacteriol 184:3466–3475

Martínez-Hidalgo P, Hirsch AM (2017) The nodule microbiome: N_2-fixing rhizobia do not live alone. Phytobiomes 1:70–82

Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J Bacteriol 149:114–122

Ohyama T (2017) The role of legume-Rhizobium symbiosis in sustainable agriculture. In: Sulieman S, Tran LS (eds) Legume nitrogen fixation in soils with low phosphorus availability. Springer, Cham, pp 1–20

Pellock BJ, Teplitski M, Boinay RP, Bauer WD, Walker GC (2002) A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. J Bacteriol 184:5067–5076

Reuber TL, Walker GC (1993) Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Cell 74:269–280

Rinaudi L, Fujishige NA, Hirsch AM, Banchio E, Zorreguieta A, Giordano W (2006) Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. Res Microbiol 157:867–875

Rinaudi LV, González JE (2009) The low-molecular-weight fraction of exopolysaccharide II from *Sinorhizobium meliloti* is a crucial determinant of biofilm formation. J Bacteriol 191:7216–7224

Rinaudi LV, Giordano W (2010) An integrated view of biofilm formation in rhizobia. FEMS Microbiol Lett 304:1–11

Simon R, Hötte B, Klauke B, Kosier B (1991) Isolation and characterization of insertion sequence elements from gram-negative bacteria by using new broad-host-range, positive selection vectors. J Bacteriol 173:1502–1508

Skorupska A, Janczarek M, Marczak M, Mazur A, Król J (2006) Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb Cell Fact 16(5):7

Sorroche F, Bogino P, Russo DM, Zorreguieta A, Nievas F, Morales GM, Hirsch AM, Giordano W (2018) Cell autoaggregation, biofilm formation, and plant attachment in a *Sinorhizobium meliloti lpsB* mutant. Mol Plant Microbe Interact 31:1075–1082

Sorroche F, Giordano W (2012) PCR analysis of *expR* gene regulating biosynthesis of exopolysaccharides in *Sinorhizobium meliloti*. Biochem Mol Biol Educ 40:108–111

Sorroche F, Rinaudi L, Zorreguieta A, Giordano W (2010) EPS II-dependent autoaggregation of *Sinorhizobium meliloti* planktonic cells. Curr Microbiol 61:465–470

Sorroche F, Spesia MB, Zorreguieta A, Giordano W (2012) A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina. Appl Environ Microbiol 78:4092–4101

Vicario JC, Primo ED, Dardanelli MS, Giordano W (2015) Promotion of peanut growth by co-inoculation with selected strains of *Bradyrhizobium* and *Azospirillum*. J Plant Growth Regul 35:413–419

Wells DH, Chen EJ, Fisher RF, Long SR (2007) *ExoR is* genetically coupled to the *ExoS-ChvI* two-component system and located in the periplasm of *Sinorhizobium meliloti*. Mol Microbiol 64:647–664

Zhan HJ, Lee CC, Leigh JA (1991) Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations. J Bacteriol 173:7391–7394

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.